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The hydrolase Lpql primes mycobacterial peptidoglycan recycling

Patrick J. Moynihan¹ , Ian T. Cadby¹, Natacha Veerapen¹, Monika Jankute¹, Marialuisa Crosatti², Galina V. Mukamolova², Andrew L. Lovering¹ & Gurdial S. Besra¹ 

Growth and division by most bacteria requires remodelling and cleavage of their cell wall. A byproduct of this process is the generation of free peptidoglycan (PG) fragments known as muropeptides, which are recycled in many model organisms. Bacteria and hosts can harness the unique nature of muropeptides as a signal for cell wall damage and infection, respectively. Despite this critical role for muropeptides, it has long been thought that pathogenic mycobacteria such as *Mycobacterium tuberculosis* do not recycle their PG. Herein we show that *M. tuberculosis* and *Mycobacterium bovis* BCG are able to recycle components of their PG. We demonstrate that the core mycobacterial gene *lpql*, encodes an authentic NagZ β -N-acetylglucosaminidase and that it is essential for PG-derived amino sugar recycling via an unusual pathway. Together these data provide a critical first step in understanding how mycobacteria recycle their peptidoglycan.

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The cell wall of *Mycobacterium tuberculosis* is built upon a foundation of peptidoglycan (PG). The remainder of this structure is formed by the modification of muramic acid residues with an arabinogalactan polymer that is in turn esterified by mycolic acids¹. This waxy coating not only contributes to drug resistance in *M. tuberculosis*, but is also the target of several mycobacteria-specific antibiotics¹. The challenge of multi- and extensively drug-resistant *M. tuberculosis* has not adequately been met by drug discovery efforts, however, recent reports suggest that β -lactams are effective in treating these drug-resistant infections^{2–4}. Despite their therapeutic promise, we know relatively little about the turnover of PG in mycobacteria, which is the eventual target of β -lactam antibiotics⁵.

For most bacteria, maintenance of a PG sacculus is an essential aspect of life. PG is a heteropolymer comprised of glycan chains with a repeating disaccharide motif of *N*-acetylglucosamine β 1 \rightarrow 4 *N*-acetylmuramic acid (GlcNAc-MurNAc), which are then cross-linked to one another via short peptides attached to the C-3 D-lactyl moiety of MurNAc (Fig. 1a). The integrity of this macromolecule must be maintained under most growth conditions and its rupture leads to lysis and cell death⁶. As a result of this essentiality, it is vital that cells are able to withstand their own internal turgor pressure and still be able to cleave the cell wall to allow for division, growth, and the insertion of macromolecular structures such as secretion systems⁶. Throughout this process, the activity of lytic enzymes or through the attack of host agents like lysozyme, the sacculus is cleaved with the resulting generation of small PG fragments⁷.

In Gram-positive bacteria, muropeptides are typically released from the cell wall through the action of lysozyme-like hydrolytic enzymes, whereas in Gram-negative bacteria, lytic transglycosylases generate 1,6-anhydroMurNAc products^{8,9}. These metabolites have been shown to be important in many aspects of host-pathogen interactions. For example, tracheal cytotoxin

produced by *Bordetella pertussis* is the product of lytic transglycosylases¹⁰. Release of a similar molecule has also been shown to be involved in tissue damage during *Neisseria gonorrhoeae* infection and in the closure of the light-organ of the bobtail squid^{11,12}. In many organisms, soluble PG acts as a potent immune stimulator once sensed by NOD receptors and other pattern recognition receptors¹³.

Aside from host organisms, PG metabolites are also important signalling molecules for the bacteria themselves. Recycling of PG has been studied in great detail in a small number of organisms including *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* among others¹⁴. The recycling pathway typically involves the step-wise degradation of the polymer into its monomeric constituents, monosaccharides, and amino acids (Fig. 1b). Despite common biochemical steps, compartmentalisation of these steps tends to be organism specific⁸. The resulting monosaccharides are eventually phosphorylated and MurNAc-6-phosphate is converted into glucosamine-6-phosphate through the activities of cytoplasmic MurQ and NagA enzymes (Fig. 1b). At the same time, the stem peptides are degraded to smaller components and typically shunted back into PG biogenesis. Growth on MurNAc as a sole carbon source has never been demonstrated for a bacterium that lacks MurQ. Furthermore, recycling of MurNAc in a bacterium that lacks MurQ has only been described in *Pseudomonas putida*, and many bacteria, including mycobacteria, are not thought to recycle their PG at all¹⁵.

In the present study, we sought to determine whether mycobacteria are capable of recycling their PG and if so, what impact this has on the bacterium. We demonstrate that these bacteria do indeed possess the biochemical capacity to recycle PG elements and determined how the bacteria generate free MurNAc for recycling. Our data indicate that loss of a key recycling enzyme, LpqI, leads to increased antibiotic and lysozyme resistance.

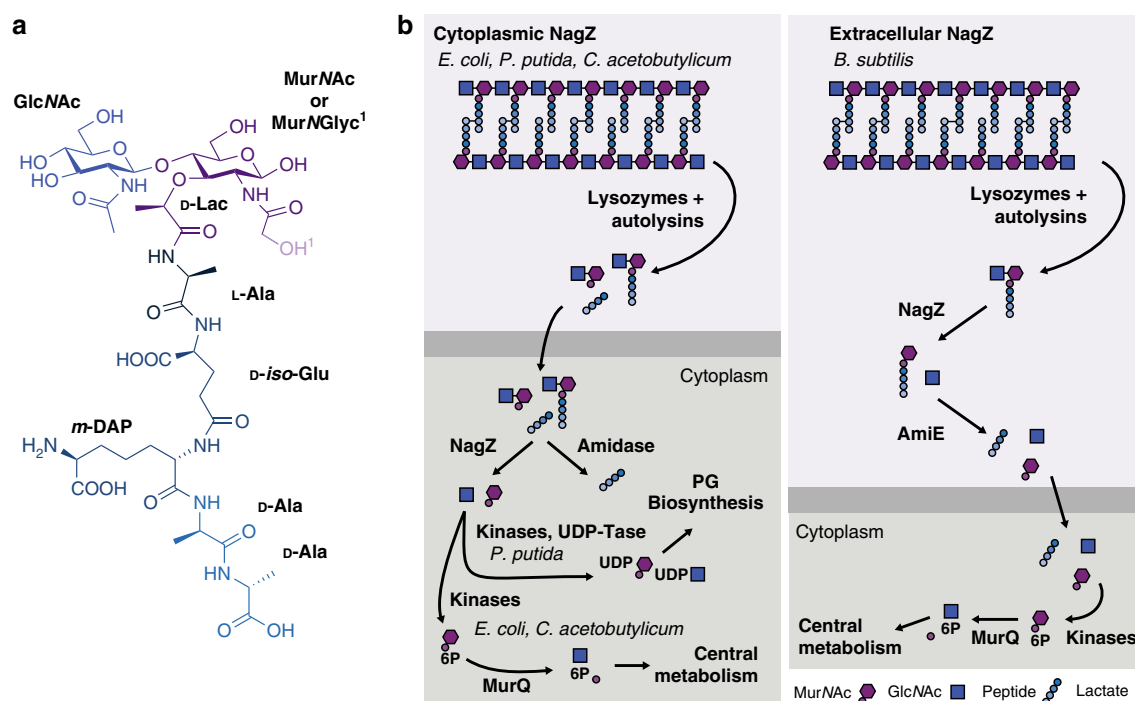


Fig. 1 Overview of PG recycling. **a** The basic building block of PG is GlcNAc-MurNAc-pentapeptide. Enzymes produced by the bacterium or the host are able to cleave every major linkage in PG. **b** Known PG-recycling machinery is variable with respect to the localisation of NagZ and the subsequent conversion to GlcNAc-1P or UDP-GlcNAc/MurNAc. All known MurNAc recovery systems that sustain bacterial growth (as opposed to strictly recycling e.g. *P. putida*) terminate at MurQ in the cytoplasm

Results

PG-recycling genes in mycobacteria. The genome of *M. tuberculosis* encodes many lytic enzymes, including at least five resuscitation-promoting factors (Rpf) and >10 peptidases and amidases in addition to penicillin-binding proteins with potential lytic activities¹⁶. The Rpf are most likely lytic transglycosylases with the product of RpfB having been recently confirmed as a GlcNAc-1,6-anhydroMurNAc disaccharide-peptide¹⁷. Although *M. tuberculosis* does appear to encode at least one lysozyme, Rv2525, its activity has not been demonstrated¹⁸. A recent comparative study of PG-active enzymes in mycobacteria indicated that although significant differences exist, enzymes that can likely degrade all of the major covalent linkages of PG are encoded in the genomes of all mycobacteria¹⁶. The products of most of these enzymes have not been experimentally demonstrated, however, their conservation underscores the importance of PG-remodelling during growth and division of mycobacteria.

Most autolytic enzymes produce small PG metabolites (muropeptides), indicating that mycobacteria should generate these molecules during the course of normal growth. Indeed, soluble PG fragment release has been observed for both *M. smegmatis* and *M. tuberculosis* in vitro^{19,20}. Given the slow release of PG fragments by mycobacteria, we evaluated the presence of known PG-recycling systems in the genome of several corynebacterial species (Supplementary Table 2)^{15,21}. BLAST analysis of the *Corynebacterium glutamicum*, *M. tuberculosis*, *Mycobacterium leprae*, and *M. bovis* BCG genomes indicates that they lack genes related to any known muropeptide import proteins, PG-metabolite phosphorylation systems, and *murQ*. The only sugar-kinase orthologs identified in the genome have previously been characterised as glucose-kinases although they have not been directly tested for amino sugar-phosphotransferase activity²². This contrasts sharply with *M. smegmatis* for which an apparently complete “classical” muropeptide recovery system exists, making it a poor model for the PG metabolism of *M. tuberculosis* (Supplementary Table 2). Taken together, the available data indicates that *M. tuberculosis* and almost all other mycobacteria lack most of the known PG-recycling genes from other bacteria, with only two conserved genes potentially associated with PG-recycling, (*nagA*—Rv3332, *nagZ/lpqI*—Rv0237).

Biochemical and structural characterisation of LpqI. In previously characterised PG-recycling systems free amino sugars are produced by NagZ, which belongs to the CAZy glycoside hydrolase family 3 (GH3)²³. This family is a large group of enzymes that have hydrolytic and phosphorytic activity and remove β -linked sugars from proteins and polysaccharides^{24,25}. The β -N-acetylglucosaminidase sub-family including all known NagZ enzymes utilise a conserved Asp-His catalytic dyad, which has been well characterised^{26,27}. A BLAST search of the *M. tuberculosis* H37Rv genome revealed only one NagZ ortholog, which was previously named LpqI in light of its identification as a lipoprotein including an appropriately positioned lipobox at the N-terminus of the protein²⁸. As a lipoprotein LpqI is expected to be found attached to the external face of the cytoplasmic membrane, which is consistent with proteomics results²⁸. LpqI has also been identified as a likely mannoseylated glycoprotein in a proteomics screen using ConA chromatography²⁹. The *lpqI* gene is found in all mycobacteria with sequenced genomes including *M. leprae*, which has a substantially reduced genome suggesting that it is involved in a conserved process across all mycobacteria (Supplementary Table 2, Supplementary Fig. 1).

Given the absence of other PG-recycling-associated genes, we sought to identify the function of LpqI. Although LpqI bears significant sequence similarity to known β -N-acetylglucosaminidases,

recent studies have demonstrated that divergent activities for this sub-family of enzymes are possible²⁵. These activities included the ability to release sugars other than GlcNAc from reporter substrates and apparent phosphorytic activity. To test this, we cloned, expressed and purified LpqI_{TB} using an N-terminal His₆-SUMO tag, which was subsequently cleaved from the protein. We first determined whether the protein was in fact a β -N-acetylglucosaminidase by testing its activity on a variety of substrates including many sugars that would be found in the cell wall of mycobacteria. Using convenient reporter sugars we assessed the ability for the enzyme to release *p*-nitrophenolate or 4-methylumbelliferone from a variety of conjugated sugars (Fig. 2a). Although not exhaustive, this set of sugars covers most major modifications to the cell wall including the AG itself, O-mannose modifications of proteins, GalN modification of arabinan, the rhamnose-linker sugar of AG and the GlcNAc and GlcN found in PG. The only detectable activity for LpqI was with GlcNAc-containing substrates (Fig. 2a). Critically, in mycobacteria, this sugar is limited to the backbone of PG and a small amount in the linker unit (MurNAc-6-P-Rha-GlcNAc-galactan) between PG and arabinogalactan. The Michaelis-Menten constants ($k_{\text{cat}} = 2.0 \times 10^{-2} \pm 0.04 \times 10^{-2} \text{ s}^{-1}$ and $K_m = 72 \pm 5 \mu\text{M}$) of LpqI using 4MU-GlcNAc as a substrate were found to be similar to other NagZ enzymes using this substrate (Fig. 2b)²⁶. In a similar assay we were also able to show that LpqI releases GlcNAc from soluble PG fragments (Fig. 2c), including PG fragments with a 1,6-anhydroMurNAc terminus (Supplementary Fig. 2). Although hydrolytic activity has been reported for most NagZ-type enzymes, a recent report suggested that β -N-acetylglucosaminidases from the GH3 family are in fact phosphorylases²⁵. Another GH3 β -N-acetylglucosaminidase was recently reported to lack this latter activity, suggesting that it may not be a general property of the family³⁰. We tested the activity of the enzyme under the same conditions as reported previously (50 mM sodium phosphate buffer, pH 7.0, 300 mM NaCl, 5 mM MgSO₄, 10% glycerol, 1 mM DTT) for Nag3 from *Celulomonas fimi* and found that there was no detectable difference with our observed hydrolytic activity. The product of the reaction also co-migrated with GlcNAc on TLCs and not GlcNAc-1-P (Supplementary Fig. 3).

To confirm that LpqI is an exo-acting enzyme rather than an autolytic β -N-acetylglucosaminidase we evaluated the activity of the enzyme using defined-length chitooligosaccharides. As shown in Supplementary Fig. 4a, LpqI produces GlcNAc₅ and GlcNAc from GlcNAc₆. This is in contrast to the known endo-acting chitinase ChitB, which produces GlcNAc₂ and GlcNAc₄ from the same substrate³¹. We further tested the ability of the enzyme to act as an autolysin and release soluble PG from insoluble material by incubating the enzyme with fluorescently labelled PG. In this assay, LpqI was not observed to release any soluble PG in contrast to the muramidase mutanolysin (Supplementary Fig. 4b). Together these data support the role of LpqI as an exo-acting enzyme involved in fragment recycling, rather than as an endo-acting autolysin.

To further confirm its function and validate its role in PG-recycling, we solved the 1.96 Å X-ray crystal structure of LpqI (PDB code: 6GFV; Supplementary Fig. 1, Supplementary Table 3). LpqI consists of a single TIM-barrel domain similar to cytoplasmic Gram-negative orthologs but lacks the C-terminal domain associated with extracellular NagZ enzymes from some Gram-positive bacteria (Supplementary Fig. 5). Alignment of LpqI with the NagZ/GlcNAc/1,6-anhydroMurNAc complex from *P. aeruginosa* (NagZ_{Pa}; PDB:5G3R) or NagZ from *B. subtilis* (PDB:4GYJ) using the SSM Superpose function in COOT resulted in a root-mean-square deviation of 2.30 Å and 1.63 Å, respectively (Supplementary Fig. 5). Superposition of the post-cleavage

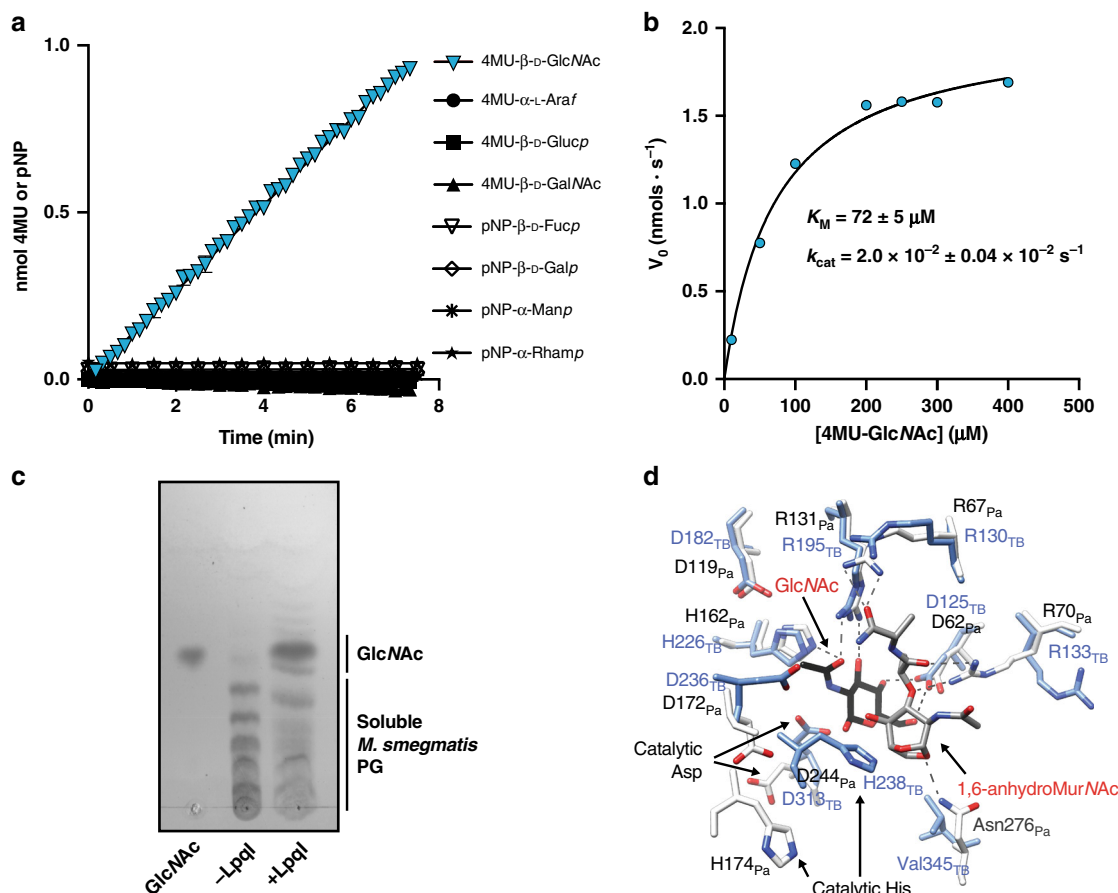


Fig. 2 LpqI is an authentic NagZ-type enzyme. **a** Reactions including 1 μM LpqI and the indicated chromogenic substrates at 1 mM were incubated at 37 °C and release of pNP or 4MU was followed by absorbance or fluorescence respectively. Cleavage of 4MU-GlcNAc is indicated by blue triangles. **b** LpqI was incubated with increasing concentrations of 4MU-GlcNAc. The rate of 4MU release was plotted and the curve fit with the Michaelis-Menten equation using GraphPad Prism 7.0. ($n = 3$, error bars are \pm SD). **c** Thin-layer chromatograph of reaction products showing that LpqI is able to release GlcNAc from soluble mucopeptides derived from *M. smegmatis* mc²155 PG. **d** The active site of LpqI is highly conserved as evidenced by the similar positioning of key binding residues observed in the GlcNAc, 1,6-anhydroMurNAc complex with NagZ_{Pa} (PDB: 5G3R) the main chains of which have been aligned with an RMSD of 2.3 Å using LSQ KAB⁵⁷

NagZ_{Pa} complex with LpqI indicates that the appropriate coordinating residues for MurNAc or 1,6-anhydroMurNAc recognition are intact in LpqI, supporting its role in PG-recycling (Fig. 2d)³².

LpqI-catalysed utilisation of PG components by mycobacteria.

Having confirmed the *in vitro* activity of LpqI, we sought to determine the fate of its reaction products, MurNAc and GlcNAc, in growing *M. bovis* BCG. Prior research has shown that most mycobacteria are unable to use GlcNAc as a sole carbon source, with *M. smegmatis* being one of the notable exceptions³³. Amino acids including D-Ala and D-Glu have previously been shown to serve as nitrogen sources for *M. tuberculosis* H37Rv but they do not serve as carbon sources³⁴. L-Ala has also been shown to not serve as a carbon source for *M. bovis*³⁵. To our knowledge, recycling of GlcNAc or MurNAc by mycobacteria has not been reported, nor has recycling been tested for soluble PG fragments. To evaluate this, *M. bovis* BCG was cultured in minimal media supplemented with glycerol (1% v/v) or MurNAc (0.2% w/v) in Sauton's minimal media with constant aeration. As observed in Fig. 3a, *M. bovis* BCG was able to grow using MurNAc as a sole carbon source. To confirm that this was not a phenotype specific to *M. bovis* BCG we also evaluated the ability of *M. tuberculosis* H37Rv to grow on the same carbon sources with identical results (Fig. 3b). Intriguingly growth on MurNAc in broth was heavily

dependent on the aeration of the culture. In contrast, growth on glycerol was unaffected by this change (Fig. 3c). To further evaluate the potential for mycobacteria to take up GlcNAc but use it for purposes other than central metabolism, we tested the ability of *M. bovis* BCG to incorporate ¹⁴C GlcNAc into whole cells. Under different growth conditions (rich medium, carbon-poor medium, aerated cultures, static cultures) we were unable to detect significant amounts of GlcNAc being taken up by *M. bovis* BCG. In all cases, the c.p.m. of the label in whole cells was less than or equal to unlabelled controls. We conclude from these data that pathogenic mycobacteria are able to utilise MurNAc, but not GlcNAc in an O₂-dependent fashion.

Mechanism of MurNAc metabolism. Given its structural similarity to GlcNAc, the ability of *M. tuberculosis* and *M. bovis* BCG to grow on MurNAc was surprising and so we evaluated the biochemical processing steps associated with MurNAc utilisation. MurNAc is a combination of GlcNAc and D-lactate joined by an ether linkage. This suggests that the bacterium is either using the GlcNAc moiety for glycolysis, or shunting the lactate derived from MurNAc into the TCA cycle. We tested this by inhibiting glycolysis with 2-deoxyglucose in cultures grown using MurNAc, glucose, and glycerol as sole carbon sources (Supplementary Fig. 6). These data suggested that the pathway of MurNAc utilisation did not require glycolysis and indicated that the lactate

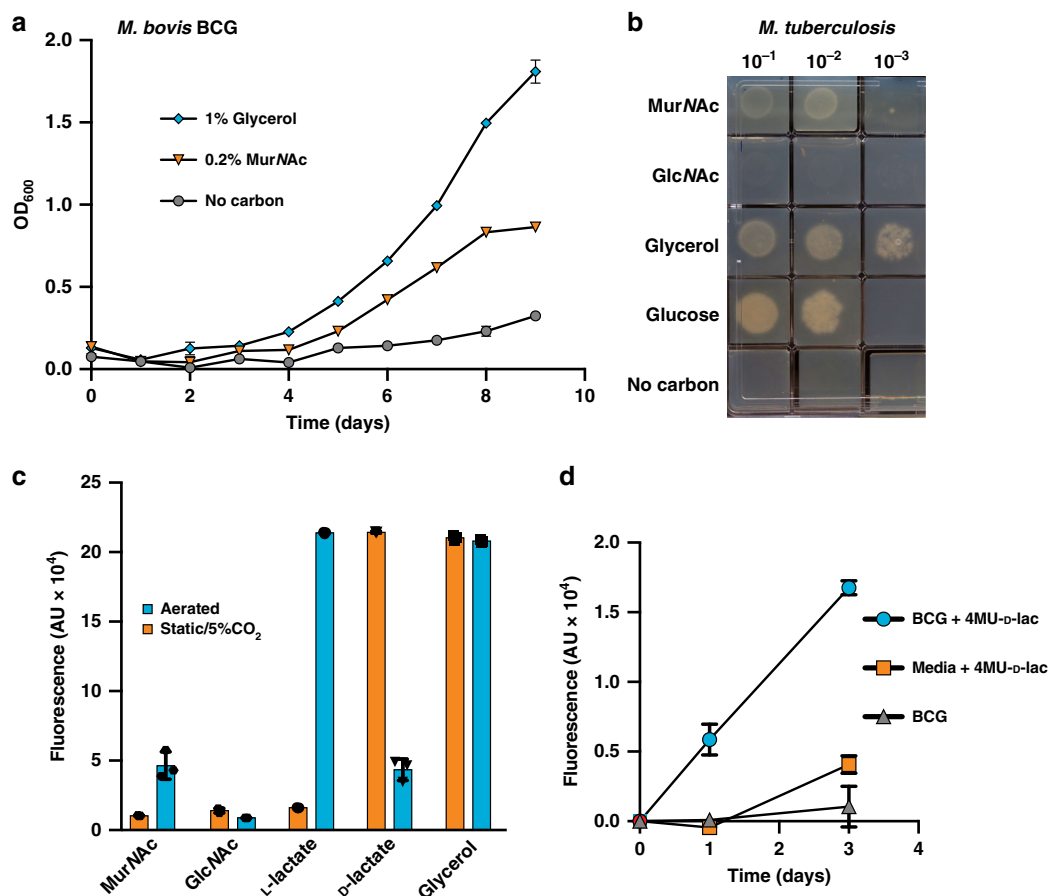


Fig. 3 *M. tuberculosis* and *M. bovis* BCG are able to recycle MurNAc. **a** *M. bovis* BCG WT was inoculated at a starting OD₆₀₀ of 0.1 in Sauton's minimal media containing glycerol (blue diamonds), MurNAc (orange triangles), or no carbon (grey circles) and growth was monitored daily by taking OD₆₀₀ readings at the indicated time points ($n = 3$; error bars are \pm SD). **b** *M. tuberculosis* H37Rv was washed and then serially diluted into fresh carbon-free minimal media. 10 μ L of each dilution was spotted onto Sauton's agar containing the indicated carbon sources at 5 mM. **c** Growth of *M. bovis* BCG on 5 mM MurNAc, GlcNAc, L-lactate, D-lactate, and glycerol was evaluated in aerated (blue) or static 5% CO₂ (orange) cultures using a resazurin assay ($n = 3$; error bars are \pm SD). **d** Mid-exponential *M. bovis* BCG was grown in minimal media with 5 mM glycerol including 1 mM 4MU-D-lactate (blue circles) with constant agitation. At the indicated times, the 4MU fluorescence of the samples was determined in a BMG Polarstar plate reader ($n = 3$; error bars are \pm SD) and compared with controls without cells (orange squares) or without 4MU-D-lactate (grey triangles)

moiety of MurNAc, was instead most likely serving as a carbon source. Consistent with this, when used as a sole carbon source, growth on L-lactate and MurNAc was O₂-dependent, whereas D-lactate was better utilised under static, 5% CO₂ culture conditions, where MurNAc could not be used as a carbon source (Fig. 3c).

These data allow us to hypothesise a mechanism by which *M. bovis* BCG metabolises MurNAc. Given that metabolism of L-lactate and MurNAc are O₂-dependent, we anticipate that use of MurNAc follows cleavage of the D-lactate from MurNAc to produce lactate and GlcNAc. In this case, the O₂ dependency on MurNAc metabolism is likely the result of an O₂-dependent lactate dehydrogenase. Consistent with this, two O₂-dependent L-lactate dehydrogenases have been identified in *M. tuberculosis* (Rv0694, Rv1872c), which are required for growth on L-lactate as a sole carbon source³⁶. Given the unusual nature of MurNAc, generation of free lactate by the bacterium would require the activity of a specific lactyl-etherase. To test for the presence of this activity in whole cells, we synthesised a 4MU-D-lactate derivative to serve as a reporter-analogue of MurNAc (Supplementary Fig. 7). Consistent with the presence of a lactyl-etherase, cultures of *M. bovis* BCG were able to release 4MU from this compound during the course of growth (Fig. 3d). Together these data support a model where mycobacteria cleave the lactyl moiety

from MurNAc by an as-yet unidentified enzyme and utilise the product of that reaction as a carbon source under aerated conditions.

LpqI-dependent uptake of PG metabolites by mycobacteria. Although our data strongly support metabolism of MurNAc by *M. bovis* BCG, we wanted to confirm the role of LpqI in mycobacterial PG-recycling. To do this we constructed a mutant strain of *M. bovis* BCG-lacking *lpqI* using specialised transduction³⁷. To validate that LpqI is the only β -N-acetylglucosaminidase produced by *M. bovis* BCG, we used a whole-cell β -N-acetylglucosaminidase assay. This demonstrated that *M. bovis* BCG Δ *lpqI* is devoid of β -N-acetylglucosaminidase activity as the amount of 4MU released is not significantly different from the spontaneous release in sterile media (Fig. 4a). This deficiency is complemented by re-introduction of the *lpqI* gene at a distal chromosomal location under the control of its native promoter (Δ *lpqI::lpqI*) and is not complemented by the empty vector (Δ *lpqI::EV*) (Fig. 4a). Growth of Δ *lpqI* in vitro is also unaltered as compared to the wild-type (Fig. 4b). This mutant therefore provided us with an opportunity to probe the role of disaccharide cleavage in mycobacterial PG-recycling.

The order in which muropeptides are recycled, and the chemical structure of the recycled material is critical for the

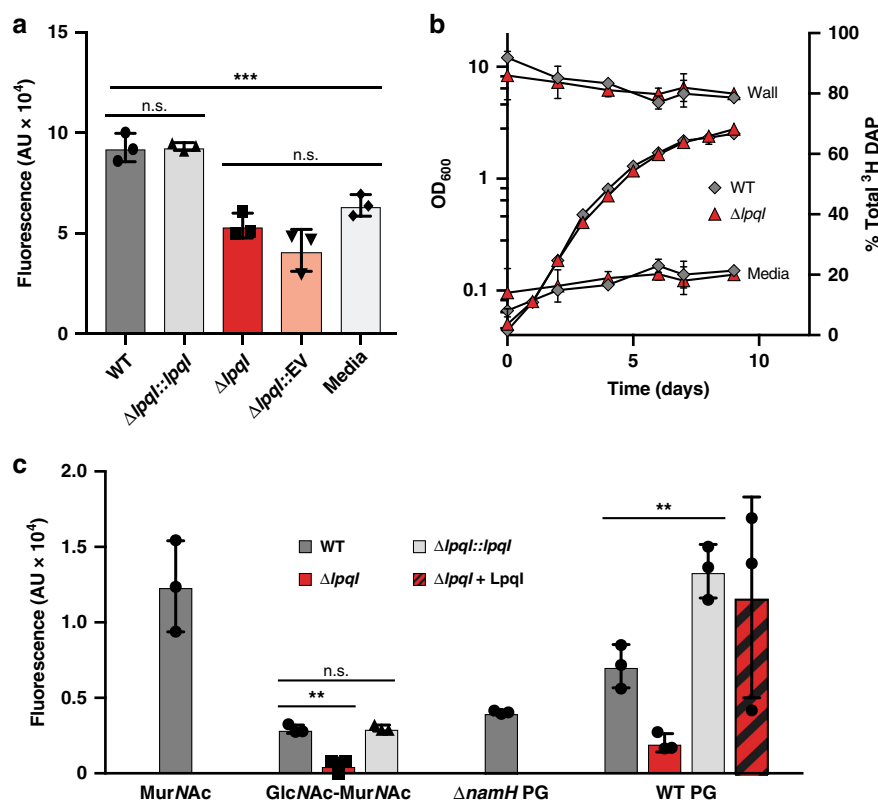


Fig. 4 *M. bovis* BCG is able to recycle PG. **a** *M. bovis* BCG WT, $\Delta lpqI$, $\Delta lpqI::lpqI$ and $\Delta lpqI::Empty Vector$ were incubated with 1 mM 4MU-GlcNAc in minimal media. After 3 days the fluorescence of the cultures were measured ($n = 3$; error bars are \pm SD). **b** *M. bovis* BCG WT (grey diamonds) and $\Delta lpqI$ (red triangles) were simultaneously evaluated for release of cell wall peptides and growth ($n = 3$; error bars are \pm SD). **c** *M. bovis* BCG WT (dark grey bars), $\Delta lpqI$ (red bars), and $\Delta lpqI::lpqI$ (light grey bars) were evaluated for their growth using MurNAc (5 mM), GlcNAc-MurNAc (2 mM, respectively), and soluble PG from *M. smegmatis* mc²155 wild-type or $\Delta namH$ (5 and 2.5 mM, respectively), and soluble PG, which had been pre-digested with Lpqi (striped bars) as sole carbon sources using a resazurin assay ($n = 3$; *** = $p < 0.001$; ** = $p < 0.005$). Statistical significance determined using a two-tailed *t*-test in GraphPad Prism 7

immune sensing of the bacterium. To determine the order of PG-recycling steps, we first assessed the impact of the loss of *lpqI* on the recycling of cell wall material. To investigate this, we generated radio-labelled muropeptides and tested them in whole-cell uptake assays. Radio-labelled muropeptides had to be generated in *M. smegmatis* mc²155 owing to the inability of *M. bovis* BCG to take up ¹⁴C GlcNAc under the conditions we tested. As shown in Supplementary Fig. 8, the *M. bovis* BCG $\Delta lpqI$ mutant took up ~25% less of the labelled PG than the wild-type (~3% vs. ~4%, respectively). In a similar assay using PG fragments labelled with ³H diaminopimelic acid (DAP) the mutant did take up slightly less of the PG than the wild-type, however this difference was not found to be significant using a two-tailed *t* test (Supplementary Fig. 8). To probe this result further, we pre-labelled cells with ³H DAP and monitored the release of the label into the culture media. Supporting the data above, we observed no significant differences between the wild-type and the $\Delta lpqI$ strain with respect to the amount of label released to the media (Fig. 4b). From these experiments, we concluded that Lpqi is involved in amino sugar recovery but is not required for stem-peptide recycling.

Given the inability of *M. bovis* BCG to take up radiolabelled GlcNAc we were unable to repeat the previous experiment using ¹⁴C GlcNAc and follow release of this sugar to the media. To unambiguously test the impact of deleting *lpqI* on amino sugar recycling by the bacterium, we evaluated its ability to grow on MurNAc, GlcNAc-MurNAc and PG from both wild-type *M. smegmatis* mc²155 and $\Delta namH$ strain, which lacks the characteristic *N*-glycosyl modification of MurNAc (Fig. 4c). The $\Delta lpqI$

strain was not deficient for growth on MurNAc, however unlike the wild-type strain it was unable to grow on GlcNAc-MurNAc and exhibited only very weak growth on soluble PG as a sole carbon source (Fig. 4c). This growth was rescued by pre-digestion of the muropeptides with Lpqi (Fig. 4c). Together these data indicate that *lpqI* is required for PG-derived amino sugar recycling in *M. bovis* BCG.

Phenotypic characterisation of a $\Delta lpqI$ mutant. As indicated above, loss of Lpqi did not alter the growth rate of the bacterium in vitro. However, given that NagZ-like proteins have been found to play a role in β -lactam sensitivity in other bacteria we sought to determine the antimicrobial sensitivity of the $\Delta lpqI$ strain. In contrast to inhibition of *P. aeruginosa* NagZ, deletion of *lpqI* resulted in an increase in survival in the presence of lysozyme and all cell wall active antibiotics tested (Fig. 5a–d)³⁸. A smaller impact on survival in the presence of the protein synthesis inhibitor chloramphenicol was observed (Fig. 5e). This increase in resistance is not owing to a change in cell wall permeability as determined by ethidium bromide uptake (Fig. 5f). These data indicate that *lpqI*-dependent amino sugar recycling is involved in the expression of antibiotic and lysozyme resistance by mycobacteria in vitro.

Discussion

In an attempt to develop diagnostic media for the identification of mycobacteria, several groups in the 1960s observed that *M. tuberculosis* and most other mycobacteria could not metabolise GlcNAc as a sole carbon source^{33,34}. This, along with the absence

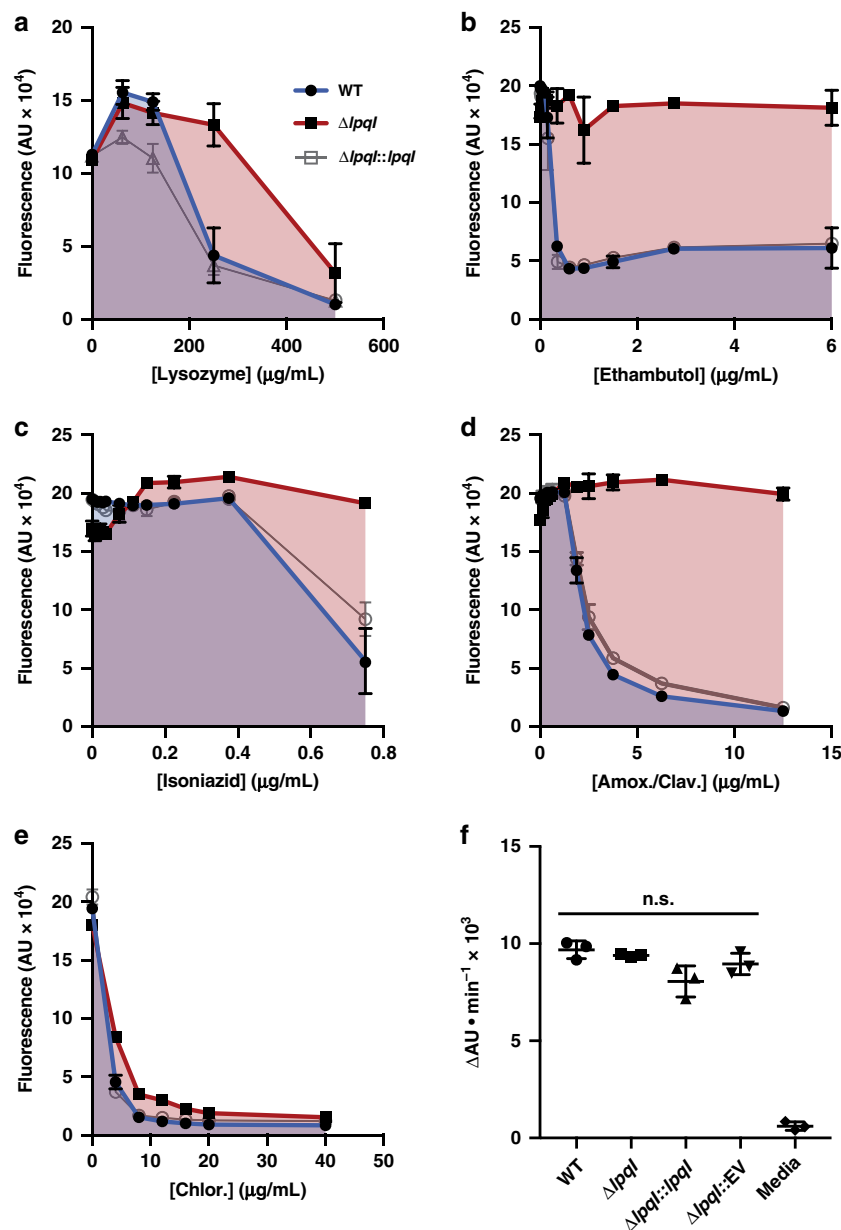


Fig. 5 Loss of LpqI leads to lysozyme and antibiotic resistance. **a–e** *M. bovis* BCG WT (blue), $\Delta lpqI$ (red), and $\Delta lpqI::lpqI$ (grey) were incubated with increasing concentrations of lysozyme or antibiotics at a starting OD₆₀₀ of 0.1. After 7 days incubation total growth was assessed using a resazurin assay, where total fluorescence correlates with respiration and growth ($n = 3$; error bars are \pm SD; Amox./Clav—amoxicillin plus clavulanic acid; Chlor.—chloramphenicol). **f** *M. bovis* BCG WT, $\Delta lpqI$ and $\Delta lpqI::lpqI$ and $\Delta lpqI::EV$ were incubated with EtBr and the rate of EtBr uptake was monitored as an increase in fluorescence. No significant differences were found in pairwise *t* tests across all strains ($n = 3$; error bars are \pm SD). Statistical significance determined using a two-tailed *t* test

of known PG recycling-associated genes lead to the assumption that PG recycling is absent in pathogenic mycobacteria. Based on our data and the literature, it is clear that not only is *M. tuberculosis* able to recycle its PG, it is generating two distinct classes of molecules. These are GlcNAc-MurNAc-peptide, which is sensed by the host, and GlcNAc-MurNAc that is sensed by the bacterium. Although host-sensing of PG is unaffected by the presence of GlcNAc on NOD-stimulatory molecules, our data indicate that LpqI acts as a regulator for GlcNAc-MurNAc levels by cleaving disaccharides and allowing the breakdown of MurNAc. In other bacteria, cell wall damage can trigger various stress responses, and so it is likely that a build-up of GlcNAc-MurNAc disaccharides may trigger a stress-like response in mycobacteria³⁹. Consistent

with this, *lpqI* is encoded adjacent to a universal stress response protein in several species of mycobacteria (Supplementary Fig. 1).

As a starting point to investigate PG recycling in *M. tuberculosis*, we characterised the core mycobacterial protein, LpqI. Despite the absence of other known PG-recycling proteins, we have shown that LpqI is an authentic exo-acting β -N-acetylglucosaminidase, which is able to cleave PG fragments in vitro. Consistent with a role in PG-recycling, *M. bovis* BCG $\Delta lpqI$ is unable to grow on soluble PG as a sole carbon source, whereas recycling of the stem-peptide is unaltered in this mutant. Together, our data demonstrate that *M. bovis* BCG and *M. tuberculosis* remove the stem-peptide from PG fragments prior to disaccharide cleavage and lactyl-ether removal (Fig. 6). The

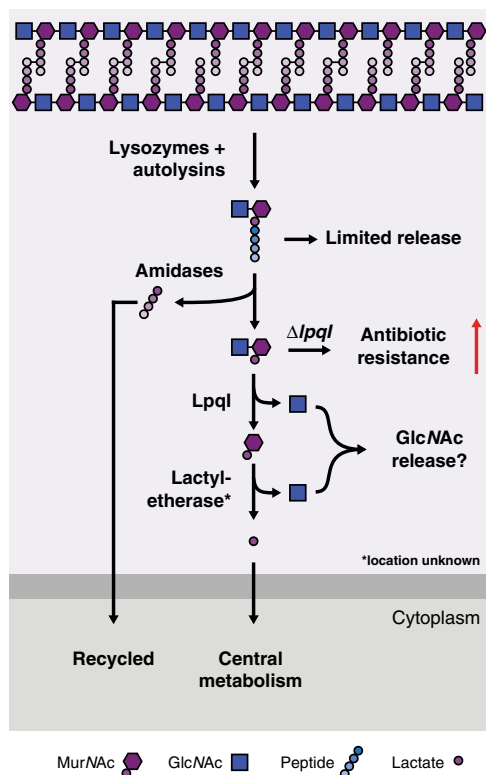


Fig. 6 Peptidoglycan recovery pathway in pathogenic mycobacteria. Based on our observations we can propose the following model for PG recycling and recovery in mycobacteria. Cleavage of the cell wall by endogenous autolysins or host-derived lysozyme generates muropeptides. Some of this material undergoes limited release to stimulate the host immune system. The remainder are subsequently degraded by amidases and other peptidases. Lpql then cleaves GlcNAc-MurNAc, which is followed by D-lactyl-ether cleavage. Lactate can then be used by the cell under aerobic conditions and GlcNAc (or its derivatives) are most likely released. Perturbation of this system by deleting Lpql leads to increased resistance to anti-mycobacterial agents

processing of GlcNAc-MurNAc by Lpql prior to lactyl-ether cleavage is also supported by our Lpql crystal structure in which the lactate-binding residue R67 from the *P. aeruginosa* structure is conserved (Lpql: R130), suggesting that the physiological substrate of this enzyme possesses the lactyl group³².

Our sole carbon source assays indicate that although the bacteria are unable to metabolise GlcNAc, surprisingly they can use MurNAc as a sole carbon source (Fig. 3). This is despite the fact that they lack an ortholog of the only known lactyl-etherase, MurQ that cleaves an otherwise stable lactyl-ether in the cytoplasm of most model organisms (Fig. 1). Our data indicate that rather than using the GlcNAc portion of the MurNAc, the bacteria are cleaving the lactyl-ether and metabolising the liberated lactate. During our study we found that *M. bovis* BCG was only able to grow on MurNAc under aerated conditions. This was also found to be the case for L- but not D-lactate, which served as a much better carbon source under O₂-limiting conditions. This suggests that L-lactate is likely the metabolised product of this reaction. The presence of a specific lactyl-etherase is further supported by the turnover of a 4MU-D-lactate reporter compound by *M. bovis* BCG. The O₂ dependence of growth on MurNAc is intriguing as N-glycosylation is also an O₂-dependent activity, suggesting significant alterations to PG metabolism in hypoxic vs. aerobically growing mycobacteria⁴⁰. Consistent with this observation, expression of *lpql* is upregulated twofold during

re-aeration after re-activation from non-replicating persistence in the Wayne hypoxia model⁴¹.

The fate of GlcNAc in this pathway remains unclear, although our data and prior observations suggest that the bacteria do not re-use this sugar. The inability to utilise this sugar is surprising given the conservation of the *nagA* (Rv3332) gene in mycobacteria⁴². However, it is possible that an alternative GlcNAc-recovery pathway exists that was not functional under the conditions we have tested. This is hinted at, with our ¹⁴C-labelled muropeptides, where incorporation of the labelled-GlcNAc is not expected, given the lack of monomeric GlcNAc utilisation by the cells. During the production of radio-labelled PG in *M. smegmatis*, a portion of the GlcNAc will have been used by the bacterium to generate MurNAc rather than strict shunting of GlcNAc into UDP-GlcNAc for cell wall biosynthesis. The subsequent removal of the lactyl-ether from this ¹⁴C-MurNAc by *M. bovis* BCG would then follow steps and intermediates we do not yet fully understand. Indeed, bacterial etherases comprise a diverse number of mechanisms and potential reaction products and so a product other than free GlcNAc is entirely possible⁴³. We are currently trying to identify and characterise the enzyme responsible for the observed lactyl-etherase activity.

In conclusion, we have identified a PG recovery pathway in pathogenic mycobacteria. We have shown that this occurs in a step-wise fashion by removing stem-peptide from PG and subsequently cleaving the PG-disaccharide and finally releasing the D-lactate from free MurNAc. Finally, we have shown that recycling of PG by these bacteria is important for lysozyme and antibiotic resistance.

Methods

Bacterial strains and growth conditions. Unless stated otherwise, all chemicals and reagents were purchased from Sigma Aldrich. *M. tuberculosis* H37Rv, *M. bovis* BCG (Pasteur), and related mutants were maintained on Middlebrook 7H10 agar or 7H9 broth supplemented with 10% OADC enrichment and 0.05% Tween 80. Appropriate kanamycin or hygromycin was added at 25 or 50 µg mL⁻¹, respectively. *Mycobacterium smegmatis* strains mc²155 and mc²155 *ΔnamH* were maintained on Tryptic Soy Broth or Tryptic Soy Agar where appropriate⁴⁰. For growth on defined carbon sources, strains were cultivated in Sauton's minimal medium containing per 1 L, 4 g asparagine, 2 g citric acid, 0.5 g K₂HPO₄, 0.5 g MgSO₄ · 7 H₂O, and 0.05 g ferric ammonium citrate, 0.05% tyloxopol and 5 mM of each carbon source unless stated otherwise⁴⁴. *E. coli* strains were grown in lysogeny broth and supplemented with kanamycin at 50 µg mL⁻¹ or hygromycin at 150 µg mL⁻¹ where appropriate. GlcNAc-MurNAc was purchased from Toronto Research Chemicals.

Cloning and purification of Lpql^{TB}. The gene encoding Lpql (Rv0237) was amplified from *M. tuberculosis* H37Rv genomic DNA using standard PCR conditions with the *lpqITBSUMOF* and *lpqITBSUMOR* primers and cloned into the T-A site of the Champion pET-SUMO expression plasmid (Invitrogen) according to the manufacturer's specifications (all primers are found in Supplementary Table 1). For production of Lpql 1 L of *E. coli* BL21 [pLpqlTB] grown in Terrific Broth to an OD₆₀₀ of 0.6, chilled to 20 °C and induced with 1 mM IPTG and grown for a further 18 h before being collected by centrifugation. Cells were resuspended in 25 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole pH 7.8 and lysed via three passages through a French pressure cell. The protein was purified using standard IMAC procedures with washes of lysis buffer, lysis buffer including 50 mM imidazole and finally eluted with 500 mM imidazole in lysis buffer. Eluted protein was dialysed exhaustively against 25 mM Bis-Tris, 100 mM NaCl pH 7.8 in the presence of recombinant Ulp1 protease, which specifically cleaves the His₆-SUMO tag. Digested protein was passed through a second IMAC column (1 mL HisTrap FF, GE Healthcare) and the flow-through fraction was found to contain pure, untagged Lpql. Purified protein was dialysed into 25 mM Bis-Tris pH 6.5, 100 mM NaCl.

Cloning and purification of RpfB. RpfB was cloned according to the same procedure as Lpql using the primers RpfBFwd and RpfBRev. Expression and purification was achieved using the same general procedure as Lpql.

Crystallography. Prior to crystallisation, Lpql was concentrated to 20 mg mL⁻¹ in 25 mM Bis-Tris pH 7.5, 100 mM NaCl. Lpql crystals were grown by the sitting-drop vapour diffusion method by mixing an equal volume of protein solution with

1.1 M sodium malonate, 0.1 M HEPES, 0.5% w/v Jeffamine ED-2001 (pH 7.0). Crystals were cryo-protected with a saturated solution of sodium malonate and flash-cooled in liquid nitrogen. X-ray data were collected at beamline I04-1 of the Diamond Light Source, Oxford. Data were auto-processed using Xia2 and general file manipulations were performed using the CCP4 suite of programmes^{45,46}. The structure was phased by molecular replacement using the unpublished *M. smegmatis* LpqI structure (PDB: 4YYF, 67% sequence identity) using the programme PHASER (TFZ of 17). The structure was subsequently auto-built in PHENIX and the remaining parts were built in COOT with further refinement using PHENIX and PDB-REDO^{47–49}.

Kinetic characterisation of LpqI. Purified LpqI was evaluated for glycoside hydrolase activity using a variety of substrates. As an initial screening assay, LpqI was incubated at 1 μ M with either 4-methylumbelliferyl (4MU) or *p*-nitrophenyl (pNP) derivatives of a variety of sugars (arabinose, galactose, galactosamine, arabinofuranose, glucose, mannose, mannosamine, glucosamine, and *N*-acetylglucosamine) in Bis-Tris pH 7.5, 100 mM NaCl at 37 °C. The release of pNP was followed by change in absorbance at 420 nm, whereas production of 4-methylumbelliferone was monitored by fluorescence (Ex. 355 nm; Em 460 nm) in a BMG Polarstar spectrophotometer. Kinetic characterisation of LpqI_{7B} was conducted using varying concentrations of 4MU-GlcNAc. The raw data were compared to standards of pNP or 4MU. All data were analysed using GraphPad Prism 7.

Substrate specificity of LpqI. To determine whether LpqI was an endo- or exo-acting enzyme we incubated 0.1 μ M of either LpqI or ChitB (Generon) with 5 mM N,N',N'',N''',N''''-hexacetylchitohexaose (GlcNAc₆) in 25 mM phosphate buffer pH 6.5. After 3 h, 30 μ L the sample was reduced with 10 mg mL⁻¹ (final) NaBH₄ for 30 min. The NaBH₄ was quenched with 20% phosphoric acid and the pH reduced to ~4. Reaction products were separated by HPLC on a Dionex UPLC system with a Thermo Hypercarb column (100 \times 2.1 mm). Separation was achieved with a linear gradient from 0.1% trifluoroacetic acid (TFA) in H₂O to 30% acetonitrile, 0.1% TFA over 15 min and peaks were detected with absorbance at 210 nm. Peaks were identified using standards of known length. To determine whether LpqI could act as an autolysin and cleave insoluble mycobacterial PG we labelled insoluble PG from *M. smegmatis* mc²155 with fluorescein isothiocyanate⁵⁰. LpqI, mutanolysin or a chitinase were incubated at a final concentration of 1 μ M overnight with 1 mg mL⁻¹ FITC-PG conjugate in 25 mM sodium phosphate buffer pH 6.5. The sample was then filtered and the fluorescence of the soluble fraction was read (Ex. 495, Em, 519) in a BMG Fluorostar plate reader.

Digestion of cell wall material with mutanolysin or RpfB was carried out overnight at 37 °C in 20 mM ammonium acetate buffer (pH 6.5) with continuous mixing. Following digestion, solubilised muropeptides were isolated using graphitised carbon solid-phase-extraction cartridges⁵¹. Purified fractions were evaporated to dryness and the concentration of reducing sugars in the pool of soluble muropeptides was assessed using the 3-methyl-2-benzothiazolinone hydrazone assay⁵². For RpfB-generated muropeptides the concentration was estimated gravimetrically using an average molecular weight of 953.41 Da for GlcNAc-1,6-anhydroMurNGlyc-tetrapeptide.

Reactions including 1 μ M LpqI_{7B} and 0.5 mM mutanolysin-derived PG fragments or 0.1 mM RpfB-derived PG fragments in 25 mM ammonium acetate buffer pH 6.5 were incubated for 18 h at 37 °C. The reactions were then evaluated by TLC (Silica 60 F₂₅₄, Merck, Germany) using a mobile phase consisting of 1-butanol, methanol, ammonium hydroxide and water at a ratio of 5:4:2:1. TLCs were stained with α -naphthol and developed by charring.

Mutant generation. To generate the Δ lpqI strain we used specialised transduction according to established protocols³⁷. A recombinant *lpqI* knockout phage was designed to replace the chromosomal *lpqI* gene using homologous flanking regions to *lpqI* with a *sacB* gene and a hygromycin resistance cassette in-between using the LL-*lpqI*BCG, LR-*lpqI*BCG, RR-*lpqI*BCG, and RL-*lpqI*BCG primers. The resulting phage was transduced into *M. bovis* BCG and transductants were selected on 7H10 agar plates containing 75 μ g mL⁻¹ hygromycin. The mutant was verified by PCR and phenotypically with 4MU-GlcNAc where loss of *lpqI* was expected to abolish turnover of this fluorescent substrate. The complemented strain was generated by incorporating the *lpqI* gene and 150 bp 5' to the start codon containing the promoter sequence into the promoter-less integrative mycobacterial shuttle vector pMV306 using primers *lpqI*BCGCompF and *lpqI*BCGCompR to generate the resulting pMV306-*lpqI* plasmid⁵³. This was electroporated into *M. bovis* BCG Δ lpqI and transformants were selected on 7H10 agar containing hygromycin and kanamycin. The complemented mutant was verified phenotypically with 4MU-GlcNAc. A control strain was also generated using the empty pMV306 vector using the same protocols.

Antimicrobial testing. Mid-exponential cultures of *M. bovis* BCG and derivative strains were diluted to OD₆₀₀ = 0.1 in fresh 7H9 media. 100 μ L of this culture was added to a 96-well plate with the addition of 1 μ L of antibiotic/lysozyme to achieve the desired final concentration as indicated. These were incubated for 7 days at 37 °C at which point 30 μ L of 0.02% w/v resazurin and 12.5 μ L of 20% Tween 80 v/v was added to the culture. This was incubated overnight at 37 °C and the

production of resorufin was determined by fluorescence (Ex. 530 nm, Em. 590 nm) using a BMG Polarstar plate reader.

Rapid purification of mycobacterial cell wall. Rapid purification of cell wall from small cultures was carried out using a modified phenol extraction protocol¹⁹. Mycobacterial cells were grown to mid-exponential phase and collected by centrifugation. These were washed with cold phosphate-buffered saline (PBS) and resuspended in PBS and the cells were lysed in a Percellys Evolution Bead Beater at 5000 rpm for 3 min. The lysate was then transferred to glass culture tubes to which 2 mL of 98% phenol was added and vortexed for 1 min. This was heated for 1 h at 70 °C, allowed to cool and the insoluble material was collected by centrifugation at 3220 \times g. The aqueous phase was removed and 4 mL of methanol was added. This was vortexed and centrifuged again. Finally, the pellet was washed three times with methanol and once with water before being frozen or used for subsequent enzymatic treatment.

Large-scale purification of PG. Purification of PG from *M. smegmatis* was achieved following established protocols⁵⁴. Six liters of *M. smegmatis* were grown with aeration to mid-exponential phase (OD₆₀₀ = ~0.6) at which point they were harvested by centrifugation, resuspended in a minimal volume of PBS and lysed by sonication. The resulting lysate was brought to 4% sodium dodecyl sulphate (SDS) and boiled under reflux for 3 h. The insoluble material was collected by centrifugation and washed with water until the SDS was completely removed (at least 7 washes) to yield mycolyl-arabinogalactan-peptidoglycan (mAGP). The mAGP was incubated for 4 days in 0.5% (w/v) KOH in methanol at 37 °C before being washed three times with methanol. The mycolic acids were extracted with three washes of diethyl ether. The phosphodiester linking the AG-PG complex was cleaved using 0.2 M H₂SO₄ and the PG was separated from the solubilized AG by centrifugation prior to neutralisation with NaCO₃ and washed with water three times. The insoluble PG pellet was sequentially digested with α -amylase (100 μ g mL⁻¹), DNase (10 μ g mL⁻¹) and RNase (5 μ g mL⁻¹) for 8 h before proteinase K (100 μ g mL⁻¹) digestion overnight at 37 °C. The PG pellet was resuspended in a minimal volume of 1% SDS and boiled under reflux for 3 h before the SDS was removed by centrifugation and washing with water (at least seven times). The resulting material was lyophilised and stored at -20 °C until it was needed. Digestion and quantitation of this material was carried out as described above.

Synthesis of 4MU-D-lactate. Instead of the 2- or 3- step protocols published for the synthesis of 4MU-D-lactate previously, we used a simplified one step method^{55,56}. In total, 1.5 g of (S)-(-)-bromopropionic acid was added to 1 g of 4-methylumbelliferone stirring in 40 mL anhydrous dimethylformamide and 0.75 g Cs₂CO₃. This was stirred at room temperature overnight and the product was extracted three times with water/ethyl-acetate and the organic phase was dried over sodium sulphate. The organic phase was then filtered and evaporated to dryness. The product was subsequently purified using silica chromatography and was dried as a crystalline white solid.

Turnover of 4MU reporter compounds by *M. bovis* BCG. To test turnover of 4MU-GlcNAc or 4MU-D-lactate by whole cells, 100 μ L of a mid-exponential culture (OD₆₀₀ = 0.6) was added to a sterile 96-well plate in Sauton's minimal media supplemented with 0.05% Tween and 1% glycerol in addition to 1 mM 4MU-D-lactate or 4MU-GlcNAc. Similar controls lacking cells or the reporter compound were included as well. This was incubated at 37 °C and mixed at 300 rpm. On the indicated days the 4MU fluorescence of the sample was read on a BMC PolarStar microplate reader with a constant gain setting.

Turnover of *M. bovis* BCG PG in vitro. Cultures of *M. bovis* BCG wild-type, Δ lpqI, and Δ lpqI:lpqI were grown to an OD₆₀₀ of 0.6 in the presence of 10 μ Ci ³H meso-DAP, at which point they were collected by centrifugation, washed three times with sterile media and diluted to 0.01 in fresh culture flasks. Periodically a sample of 0.5 mL was taken, and the cells were collected by centrifugation. The spent medium was mixed with 10 mL scintillation fluid and counted using a liquid scintillation counter. The cell pellet was resuspended in 10% SDS, boiled for 20 min, and centrifuged again. The cell wall material was then resuspended in 1 mL scintillation fluid and the material was counted in a liquid scintillation counter. The counts of the cell wall and the media were added together to give total ³H DAP in each culture and the data is presented as a percentage of that total. During the course of the experiment the OD₆₀₀ of the culture was monitored daily. All measurements are from three biological replicates.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The atomic coordinates and structure factors of LpqI are available from Protein Data Bank with accession code 6GFV. All data used to generate the figures are available in the Source Data file. Other data that support the findings of this study are available from the authors on request.

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Author contributions

Conceived of the study: P.J.M. conceived and designed the experiments: P.J.M., G.V.M., A.L.L., G.S.B. performed the experiments: P.J.M., I.T.C., N.V., M.J., M.C. analysed the data: P.J.M., I.T.C., N.V., M.C. G.V.M., A.L.L., G.S.B. wrote the manuscript: P.J.M. edited the manuscript: P.J.M., G.V.M., A.L.L., G.S.B.

Additional information

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